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ELECTROPHYSIOLOGICAL AND BEHAVIORAL RESPONSES OF THE BLACK-BANDED OAK BORER *Coroebus florentinus* TO CONSPECIFIC INSECT AND HOST PLANT VOLATILES

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Abstract - Studies designed to investigate the chemical ecology of the black-banded oak borer (BBOB) *Coroebus florentinus* (Coleoptera: Buprestidae) are presented for the first time. The volatile composition of male and female odors did not show qualitative nor quantitative differences. Nonanal, decanal, and geranylacetone, identified from the headspace volatiles of both sexes, were clearly active on male antennae in electroantennographic assays, but scarcely on females. In dual-choice olfactometer experiments, blend of these compounds was attractive to both sexes, with males responding particularly to decanal alone and females to geranylacetone suggesting that these two compounds are the responsible chemicals for activity in the blend for each sex. These results emphasized obvious differences in the volatile perception of males and females. Antennae of both sexes displayed GC-EAD responses to the green leaf volatiles (GLVs) (*E*)-2-hexenal, (*E*)-2-hexenol, 1-hexanol, (*Z*)-3-hexenyl acetate, and *n*-hexyl acetate, identified from the host plant *Quercus suber*. In behavioral experiments, only females were attracted to the host plant odors, particularly to (*E*)-2-hexenol, 1-hexanol, and (*Z*)-3-hexenyl acetate, suggesting that these compounds could play an important role in the foraging and/or oviposition behavior of BBOB females.

Key Words - *Coroebus florentinus*, Black-banded oak borer, *Quercus suber*, Cork pest, Semiochemicals, Green leaf volatiles, Aggregation behavior, Kairomone, Electrophysiology, Y-tube olfactometer, Coleoptera, Buprestidae.

INTRODUCTION

Chemical signals produced by Coleoptera have been the subject of numerous studies focusing on their occurrence, biosynthesis, and biological significance (Francke and Dettner, 2005). Despite this, some Coleopteran families have received little attention, particularly Buprestidae. The exception is the great number of laboratory and field studies reported on the emerald ash borer (EAB), *Agrilus planipennis* (Fairnaire) (Coleoptera: Buprestidae), an invasive species originating from Asia that has caused considerable mortality of ash trees in the US and Canada (Bartelt et al., 2007; De Groot et al., 2008; Francese et al., 2008; Lelito et al., 2008; Lelito et al., 2009; Silk et al., 2009; Crook and Mastro, 2010; Silk et al., 2011), and some field experiments to catch different jewel beetles (Montgomery and Wargo, 1983; McIntosh et al., 2001). The black-banded oak borer (BBOB) *Coroebus florentinus* (Herbst) (Coleoptera: Buprestidae), together with its sister species *C. undatus*, produce notable damage to cork trees, thereby reducing the production of high quality cork. The cork oak (*Quercus suber* L.) is a valuable endemic plant of the Mediterranean region with a worldwide surface of more than 2.5 million Ha (Institute C.M.C. 1999, http://www.iprocor.org), mainly distributed over Portugal and Spain (Soria et al., 1992; Borges et al., 1997). Its great economic interest derives from the approximately 340,000 tons of cork, worth some 1.5 billion US dollars, produced annually (http://www.realcork.org), from which wine stoppers are the most visible and most profitable product with an estimated 13 billion stoppers being produced every year (http://www.corkqc.com).

Just after eclosion, BBOB larvae start feeding inside the bark of young and healthy branches of the tree, making galleries. The adults emerge around the summer solstice, feed in groups on *Quercus spp.* foliage and live 2–3 weeks before mating and egg-deposition. The insect is not lethal to standing trees but its attack provokes dryness and yellow leaves, finally resulting in the death of branches and shoots (Soria and Ocete, 1993; Lombardero and Fernández de Ana Magán, 1996). No effective control treatments have been established to date as the larvae feed underneath the bark, thus making control with insecticides impractical. Moreover, application of any type of chemical insecticide could seriously contaminate cork with the consequent severe risk to humans.

It has been shown that mate location in buprestids can be facilitated by host selection followed by visual or tactile cues (Carlson and Knight, 1969; Gwynne and Rentz, 1983). For example, the EAB is thought to use host plant volatiles for host location

(Rodriguez-Saona et al., 2006; Grant et al., 2010) and visual cues rather than long-range sex pheromones for mate finding (Lelito et al., 2007; Lelito et al., 2008). However, other investigations resulted in the discovery of two hydrocarbons as female contact pheromones, which are probably involved in mate recognition (Lelito et al., 2009; Silk et al., 2009). Moreover, a recent study showed that a volatile pheromone produced by the EAB in combination with foliar volatiles was highly attractive to conspecific individuals (Silk et al., 2011). In the BBOB, male antennae are much longer and contain more olfactory *sensilla basiconica* than those of females (unpublished), which could suggest also a likely role for a sex pheromone in this species. Herein we present initial studies to disclose the so far unknown chemical ecology of the BBOB with the final aim of developing an environmentally friendly approach to control this pest.

METHODS AND MATERIALS

Chemicals. The reference compounds for the electrophysiological and olfactometric assays, namely nonanal (95%), decanal (99%), (*E*)-2-hexenal (98%), (*E*)-2-hexenol (96%), 1-hexanol (98%), (*Z*)-3-hexenyl acetate (98%), *n*-hexyl acetate (99%), and dodecyl acetate (97%), were purchased from Sigma-Aldrich Química (Madrid, Spain). Geranylacetone [(*E*)-6,10-dimethyl-5,9-undecadien-2-one], containing 40% nerylacetone, was obtained from TCI Europe (Zwijndrecht, Belgium). *n*-Hexane (analytical purity, >95% by GC) (J.T. Baker, Deventer, Holland) was used as solvent. Isopropyl *n*-dodecanoate was obtained by esterification of dodecanoic acid with isopropyl alcohol in refluxing sulfuric acid. The product was obtained as colorless oil in 99% yield and 97% purity by GC, and was characterized by IR, ^{1}H NMR, ^{13}C NMR and MS.

Insects. Live adult BBOB specimens were obtained directly from woods in the provinces of Girona (NE Spain), Valencia (E), and Madrid (Center), because so far rearing of the insect has been unsuccessful. Cork oak trees affected by insect attacks (branches visible at the treetop with yellowish leaves) were identified at the end of May in the period 2005–2010. Branches (700–1000 per year, ca. 30 cm long × 4 cm diameter), supposedly containing at least one pupae, were placed in cardboard boxes (80 x 40 x 40 cm), half of which were maintained at 5–9 ºC, 50–70% RH for 4 weeks to retard emergence of the adults. This allowed us to work with living insects for a longer period since the life expectancy of BBOB is only around 3 weeks. The remaining

electron impact (EI) at 70 eV at a source temperature of 200 ºC. The mass range was 40–500 *m*/*z* at a scan time of 1.0 s and the solvent delay was 4.0 min. Separately, 1 µL of a C_8–C_{25} hydrocarbon mixture in hexane (100 ng μL^{-1}), containing a series of odd- and even-numbered *n*-alkanes, was injected to calculate the retention indices (RIs) of the detected compounds, as described previously (van den Dool and Kratz, 1963). Compounds were identified by comparison of their MS and RIs with those of authentic standards and/or by comparison with those from a commercial library (NIST Registry of Mass Spectral Data, 2005; Wiley, 2000), or from the database published by Adams (2007). Individual compounds were quantified by comparison of their peak areas with regard to that of the internal standard.

Abdominal Extracts. The abdomens of three 7-day-old virgin males and females were excised beneath the thorax and immersed in 500 µL of hexane in 5 mL glass vials. After extraction for 3 h at room temperature, the abdomens were removed and the extracts stored at –20 ºC for subsequent analysis and bioassays. GC-MS analyses were performed by injecting 1 µL of the extracts (not concentrated) and 100 ng of the IS, as described above.

Collection and Analysis of Host Plant Volatiles. Volatiles were collected by placing 5-6 freshly-cut *Q. suber* branches (ca. 20 cm long, fresh weight ca. 35 g) into a 3 L Erlenmeyer flask. Charcoal-filtered compressed air (550 mL min^{-1}) was passed over the branches and the volatiles adsorbed into a Porapak Q cartridge (50/80 mesh, 150/175 mg) (Supelco, Bellefonte, PA, USA). Collection (two replicates) lasted 24 h, after which the adsorbed products were eluted with 2 mL of hexane. One µl of the extracts (without concentration) and 1 µl of dodecyl acetate in hexane (100 ng μl^{-1}) as IS were injected into GC-MS and analyzed as described above. Linear RIs were estimated by co-injection of a hydrocarbon mixture (C_8-C_{25}), as described previously. The oven temperature was initially set at 50 °C for 1 min, programmed at 3 °C min^{-1} to 120 °C, then at 5 °C min^{-1} to 200 °C, 10 ºC min^{-1} to 260 ºC, and held at this temperature for 10 min more. Compounds were identified by comparison of their MS and RIs with those of authentic reference compounds and/or with MS data from the literature (Adams, 2007) or the NIST library. For quantification purposes, the percentage of each component was calculated relative to the most abundant compound.

Leaf Extracts. Five or six leaves (ca. 500 mg) from fresh *Q. suber* branches were cut into small pieces, placed into 5 mL glass vials, and immersed in hexane (3 mL). After extraction for 3 h, the plant material was filtered through Whatman paper and the

branches were brought to the laboratory and placed in light-sealed cardboard boxes (50 x 40 x 40 cm) provided with a removable glass container (11 cm long × 7 cm diameter), fixed in the middle of the front wall with a perforated lid. The light entering through the glass attracted the emerging adults from inside the box. Glass containers were checked daily and the insects collected and sexed. The sexes were distinguished based on a) the body size: females (1.5 cm long) are up to 1.2-fold larger than males, and b) the length of the antennae: male antennae (0.4 mm) are typically twice as long as those of females. The emergence of adults that had been kept at room temperature as pupae was around 60%, whereas this value decreased to 40% for pupae maintained in the cold. Adults were kept separately by sex in square glass containers (15 x 15 cm) with a removable plastic lid with wire gauze on top at 26 ± 2 ºC, 50 ± 10% RH, and a 14:10 L:D photoperiod. Fresh *Q. suber* leaves (cork oak foliage) were provided to the insects every 2 days for feeding. Beetles aged 8–18 days were used in the bioassays when they were supposed to be "mature", as described for EAB (Lelito et al., 2009).

Collection and Analysis of Insect Volatiles. A charcoal-filtered air flow (350 mL min^{-1}) was passed over male (N=20) and female (N=20) adults, placed separately in a glass trapping chamber (15 cm length, 3 cm O.D.) for 24 h at room temperature. Odor collections from empty trapping chambers were conducted as blanks and experiments with and without insects were replicated 5 times. The volatiles were trapped on Porapak Q-containing volatile collection tubes (150/175 mg, 50/80 mesh) (Supelco, Bellefonte, PA, USA), placed at the outlet of the trapping chamber. Volatile extracts were obtained by washing the absorbent with 2 mL of analytical grade hexane. Each extract contained 480 beetle/h equivalents and was stored at –20 ºC for chemical analysis and electrophysiological assays. For GC-MS analysis, 100 µL of the extracts was concentrated under a gentle nitrogen stream to a volume of around 1–2 µL to which 1 µL of a 100 ng μL^{-1} solution of dodecyl acetate in hexane was added as internal standard (IS). The final volume of the extracts was injected in splitless mode into a Thermo Finnigan Trace 2000 GC system coupled to a Trace MS quadrupole mass spectrometer (ThermoFisher Scientific, Madrid, Spain). Helium (1 mL min^{-1}) was the carrier gas and the column used was a HP-5MS (5% phenyl methyl siloxane) (30 m × 0.25 mm I.D. × 0.25 µm) (Agilent Technologies, Madrid, Spain) under the following chromatographic conditions: injection at 60 ºC, held for 5 min and programmed at 5 ºC min^{-1} to 280 ºC, which was maintained for 10 min. The column effluent was ionized by

extracts stored at –20 ºC for subsequent analysis and bioassays. GC-MS analyses were performed as described above after injection of 1 µL of the non-concentrated samples containing 100 ng of the IS. The temperature program was the same as for the plant volatiles.

Electrophysiological Responses. Coupled gas chromatography-electroantennographic detection (GC-EAD) analyses were carried out using a Focus GC (Thermo Instruments, Barcelona, Spain) equipped with a FID detector and a 30 m × 0.25 mm ID × 0.25 µm HP-5MS fused silica capillary column (Agilent Technologies, Madrid, Spain) with helium as carrier gas (1-2 mL min^{-1}) and nitrogen as a second make-up gas. The column effluent was split 1:1 for simultaneous detection by both detectors (FID and EAD). The transfer tube to the EAD preparation was heated to 230 °C, and the GC conditions were the same as for the GC-MS analysis (see above). The outlet for the EAD was delivered to the insect antenna through an L-shaped glass tube (12 cm × 6 mm ID) in a humidified airstream. For the antennal preparation, antennae of both sexes were excised, slightly cut at both ends, and the distal and proximal segments placed in contact with the microelectrodes using a conducting gel (Spectra 360, Parker Lab. Inc., Hellendoorn, The Netherlands). The microelectrodes were connected to an IDAC-2 interface (Syntech, Kirchzarten, Germany) and the antennal and FID signals were amplified (100x), filtered (DC to 1 kHz), and recorded simultaneously using the GC-EAD v4.4 software (Syntech). A 2 µL aliquot of the BBOB headspace volatile extracts, host plant volatiles, and a mixture of nonanal, decanal, and geranylacetone (100 ng $µL^{-1}$ each), detected in the headspace volatiles of adults, was injected in splitless mode. The extracts were tested on male and female antennae and a compound was considered electrophysiologically active when it elicited responses at least 3x higher than the background noise.

Behavioral Bioassays. A vertically posted, dual-choice, Y-shaped glass olfactometer was used to test the olfactory responses of BBOB males and females to conspecific insect volatiles, synthetic compounds identified from them (nonanal, decanal and geranylacetone, 100 ng and 1 µg each) and host plant volatiles ((*E*)-2-hexenal, (*E*)-2-hexenol, 1-hexanol, (*Z*)-3-hexenyl acetate and *n*-hexyl acetate; 1 µg each). The olfactometer consisted of a main tube (10 cm long × 18 mm OD) with two 8 cm arms separated at a 90º angle. The Y-tube contained an iron wire to facilitate locomotion of the insects up to the far ends of the olfactometer, according to the design used by

Sabelis and van de Baan (1983). In odor-vs-blank experiments each arm was connected to a glass adaptor (4.5 cm long × 1.2 mm ID) containing the test (3 live insects, abdominal, leaf and host plant volatile extracts, and synthetic compounds) and control stimuli (air or solvent). Males were also tested in competitive experiments, in which 100 ng of two different odorants (3-component blend vs decanal and nonanal vs decanal) were pitted against each other in the two arms of the Y-tube. The corresponding amounts of the synthetic compounds or 100 µL of each extract were deposited on a Whatman filter paper (1.5 cm diameter), placed inside the test adaptor. In the other arm, the same amount of solvent was used as control. Charcoal-filtered air (2.5 L min^{-1}) was passed through the arms to present the stimuli to the test insects, placed at the entrance of the main tube. The system was lit by a 60 W white light bulb to ensure homogenous illumination around the olfactometer. All experiments were carried out under similar conditions on 1-2 week-old adults from 10:00 to 17:00, time of displaying activity like feeding and mating (B.F., personal observations), at 26 ± 2 ºC and 50 ± 10% RH. Insects were taken out from their containers 2 h before the tests, kept individually in plastic Petri dishes (3 cm high × 5 cm OD) and placed at the base of the main arm. Individuals that walked upwind and reached at least the middle of one side arm without returning to the intersection within 5 min were recorded as a positive response; those that did not choose either arm were excluded from the analysis.

A minimum of 30 insects for each treatment was considered for analysis. The number of individuals tested in control assays and in females vs males experiments were combined from different years leading to a total of N=50-74. Assays for each treatment were implemented on at least 3 different days testing a minimum of 10 individuals per day. After 5 replicates of the same treatment, the Y-tube and the iron wire were cleaned with ethanol or acetone, left to dry and the relative position of the olfactometer arms was reversed to exclude possible position effects. Because no rearing system has been developed so far, insects were recycled for testing but never for the same treatment and only after 3 days from the previous experiment. For statistical analysis, the number of insects responding to the different stimuli was subjected to a χ^2 goodness-of-fit test, using the Yates correction for continuity (Zar, 1999). Additionally, the number of insects choosing one of the arms was subjected to a binomial test of proportions using the probability mass function to avoid that the different sample sizes of the experiments (N values) could affect the results. The null hypothesis was that the percentage of

individuals choosing the odor treatment and the blank was equal to 50%. For comparison between different odor treatments and sexes within a figure, the ratio test/control of the data was subjected to analysis of variance (one-way ANOVA) and means were compared for significance by Tukey's Honestly Significant Difference (HSD) test. In competitive experiments testing two odors against each other the resulting ratios of odor treatment I vs odor treatment II were analyzed by one-way ANOVA followed by Student's t-test. In all statistical analyses a 5% probability level was applied using PASW statistics 18.

RESULTS

Volatile Composition. A total of 28 compounds were identified in abdominal extracts and headspace volatiles of male and female adults (Table 1). Most of the identified compounds were saturated linear or branched hydrocarbons. Several carboxylic acids (entries 7, 13, and 14), 2-phenylethanol (entry 5), and nonanal (entry 4) were also detected in the abdominal samples. The aldehydes (entries 1, 3, 4, and 6), ketones (compounds 2 and 10), and two isopropyl esters (entries 12 and 15) were mostly identified in insect volatiles. No qualitative differences were observed in the headspace composition of male and female volatiles, and only slight differences when compared quantitatively. The amount of the active compounds, detected in the volatile collections from 20 individuals during 24 h was for nonanal 249.2 ± 83.8 ng in females and 388.5 ± 167.2 ng in males; for decanal 349.2 ± 107.3 ng in females and 369.1 ± 155.2 ng in males; and for geranylacetone 56.2 ± 32.1 ng in females and 122.5 ± 68.6 ng in males. In the blank collections, several phthalates and siloxanes were detected along with minute amounts of nonanal (12.3 ± 5.7 ng) and decanal (18.0 ± 9.6 ng).

Regarding host plant odors, 32 compounds were identified from the headspace volatiles of freshly cut branches and leaf extracts of *Q. suber* (Table 2). The host volatile composition essentially contained two groups of compounds, namely saturated and monounsaturated short-chain aldehydes, alcohols and esters, the so-called green leaf volatiles (GLVs), and monoterpenes. In addition, the homoterpene (3*E*)-4,7-dimethyl-1,3,7-nonatriene and some sesquiterpenes, esters, and aldehydes were also identified. The most abundant compounds were the GLVs (*E*)-2-hexenol, (*E*)-2-hexenal, and (*Z*)-3-hexenyl acetate, followed by 1-hexanol and (*Z*)-3-hexenol. The relative amounts of all

components with regard to the most abundant chemical (*E*)-2-hexenol are displayed in Table 2.

GC-EAD Recordings. On male antennae three EAD-active compounds were detected in female and male volatiles. Because both extracts were apparently identical in composition, as cited above, only the responses elicited by female volatiles are shown in Figure 1a. The active chemicals, numbered as in Table 1, were identified as nonanal (comp. 4), decanal (comp. 6), and geranylacetone (comp. 10). Activity of these compounds was confirmed using a 3-component blend of the synthetic compounds (100 ng each) (Fig. 1b). A fourth EAD-active chemical in the synthetic mixture was identified as nerylacetone (NA), the *cis* isomer of geranylacetone contained in the commercial sample of the latter. All four compounds elicited only slight responses on female antennae (data not shown).

GC-EAD analyses of the headspace volatiles from the host plant *Q. suber* indicated that most of the major GLVs identified, namely (*E*)-2-hexenal (comp. 2, Table 2), (*E*)-2-hexenol (comp. 4), 1-hexanol (comp. 5), (*Z*)-3-hexenyl acetate (comp. 6), and *n*-hexyl acetate (comp. 7), elicited responses in the antennae of both sexes (Fig. 2a-b). The homoterpene (3*E*)-4,7-dimethyl-1,3,7-nonatriene (comp. 23) was only active on female antennae.

Laboratory Behavioral Bioassays.

Live Insects and Abdominal Extracts. A preliminary test in the Y-tube olfactometer with no odor treatment (air vs air) showed that there was no preference in the response of adult males (χ^2=0.813; *df*=1; *P*>0.05; $P_{binomial}(x\leq23)$=0.288) and females (χ^2=0.071; *df*=1; *P*>0.05; $P_{binomial}(x\leq23)$=0.336) for either of the two arms, thereby indicating a lack of positional effect of the experimental setup. The responsiveness of test individuals in the bioassays was almost 100%. The results revealed that adult virgin males were significantly attracted to 3 live females (χ^2=13.549; *df*=1; *P*=0.001; $P_{binomial}(x\leq20)$=0.001) and to abdominal extracts of 3 females (χ^2=5.184; *df*=1; *P*=0.023; $P_{binomial}(x\leq12)$=0.017), but not to 3 live males (χ^2=0.567; *df*=1; *P*>0.05; $P_{binomial}(x\leq23)$=0.292). In contrast, adult virgin females were not attracted to volatiles of either sex or abdominal extracts of males and females (Fig. 3).

Synthetic Compounds. The behavioral responses of male and female adults to 100 ng of the synthetic compounds nonanal, decanal, and geranylacetone and a blend of them in 1:1:1 ratio are presented in Figure 4. Males were significantly attracted to the 3-component blend (χ^2=6.444; *df*=1; *P*=0.011; $P_{binomial}(x\leq14)$=0.008) and to decanal (χ^2=3.935; *df*=1; *P*=0.047; $P_{binomial}(x\leq10)$=0.035). In contrast, virgin females displayed the strongest attraction to the 3-component blend (χ^2=4.925; *df*=1; *P*=0.027; $P_{binomial}(x\leq13)$=0.019), and particularly to geranylacetone (χ^2=6.567; *df*=1; *P*=0.01; $P_{binomial}(x\leq8)$=0.008). At the dose of 1 µg, males (N=30) significantly chose the arm treated with the 3-component blend (22 individuals, 73% of response) over the blank (8 individuals, 27% of response) (χ^2=6.567; *df*=1; *P*=0.01; $P_{binomial}(x\leq8)$=0.008), whereas females (N=40) were attracted to the arm containing nonanal (27 individuals, 67.5 % of response) over the blank (13 individuals, 32.5% of response) (χ^2=4.925; *df*=1; *P*=0.027; $P_{binomial}(x\leq13)$=0.019). The other compounds did not evoke attraction.

In additional experiments pitting two odor treatments against each other in the two arms of the Y-tube (decanal vs 3-component blend and decanal vs nonanal), males (N=30) did not demonstrate preference between 100 ng of decanal (13 individuals, 43% of response) and the 3-component blend (17 individuals, 57% of response) (χ^2=0.567; *df*=1; *P*>0.05; $P_{binomial}(x\leq13)$=0.292), whereas they favored decanal (21 individuals, 70% of response) over nonanal (9 individuals, 30% of response) significantly (χ^2=4.833; *df*=1; *P*=0.028; $P_{binomial}(x\leq9)$=0.021) (Figure 5). The ratio decanal/nonanal was significantly higher than the ratio decanal/3-component blend (Student's t-test, *F*=12.413; *df*=4; *P*≤0.001).

Host Plant Volatiles and Synthetic GLVs. When adults of both sexes were exposed to headspace volatiles and leaf extracts from the host plant *Q. suber*, females were found to be significantly attracted to each odor source ($\chi^2_{leaf\ extract}$=3.935; *df*=1; *P*=0.047; $P_{binomial}(x\leq10)$=0.035); $\chi^2_{volatiles}$=4.833; *df*=1; *P*=0.028; $P_{binomial}(x\leq9)$=0.021). In contrast, males were not lured by either one (Fig. 6). Furthermore, females, but not males, displayed also a significant attraction in response to a mixture of the synthetic GLVs (*E*)-2-hexenal, (*E*)-2-hexenol, 1-hexanol, (*Z*)-3-hexenyl acetate, and *n*-hexyl acetate (1 µg each) (χ^2_{GLVs}=5.484; *df*=1; *P*=0.019; $P_{binomial}(x\leq9)$=0.015) (Fig. 6). Comparison between the odor/blank ratios of the different treatments within each sex by Tukey's HSD test revealed no differences, except the GLV blend which elicited a significant lower response in males. When the GLVs were tested individually, females

were significantly attracted to the saturated and unsaturated C_6-alcohols 1-hexanol and (*E*)-2-hexenol ($\chi^2_{1\text{-hexanol}}$=6.567; *df*=1; *P*=0.01; $P_{\text{binomial}}(x\leq 8)$=0.008; $\chi^2_{(E)\text{-2-hexenol}}$=3.935; *df*=1; *P*=0.047; $P_{\text{binomial}}(x\leq 10)$=0.035) and to *(Z)*-3-hexenyl acetate (χ^2=4.833; *df*=1; *P*=0.028; $P_{\text{binomial}}(x\leq 9)$=0.021), but not to *n*-hexyl acetate and (*E*)-2-hexenal (Fig. 7). Multiple comparison between the GLVs revealed that 1-hexanol was significantly more attractive than (*E*)-2-hexenal and *n*-hexyl acetate, but response towards (*E*)-2-hexenol was not statistically different to that of the other odors tested (Fig. 7).

DISCUSSION

No qualitative but small quantitative differences in composition were found in the headspace volatiles of both sexes. In behavioral bioassays using abdominal extracts as stimuli, only males showed responsiveness towards female odors, pointing out to the possible presence of a sex pheromone produced by females. The presence of differential, female-specific compounds in only trace amounts would account for these results, but further complementary experiments should be carried out to test this assumption. Because females were not attracted to the corresponding male odors, we could expect BBOB males to display a stronger affinity than females for olfactory cues produced by the opposite sex. In this regard, SEM analysis revealed that male and female antennae are nearly identical in its structure, but male antennae (ca. 4.5 mm long) are up to 2x longer and contain more olfactory *sensilla basiconica* than those of females (unpublished). This underlines that BBOB males may be more sensitive to beetle-produced volatiles than females in agreement with the above-mentioned behavioral assays.

Among the compounds identified in headspace collections, nonanal, decanal, and geranylacetone elicited GC-EAD responses in male antennae, particularly decanal which evoked the strongest antennal response. Females responded only slightly to these compounds. It should be noted, as cited above, that the two aldehydes were also detected in blank odor collections but in minute amounts in comparison with those found in volatiles of BBOB adults. Nonanal and decanal have been described as contaminants in control volatile collections of male and female flowers of *Breynia vitis-idaea* (Svensson et al., 2010) but also as active compounds emitted by plants and insects (Zhang, et al., 2003; Torto, et al., 2005; Siljander et al., 2008).

In addition to the electrophysiological activity, some of the compounds were also behaviorally active in Y-tube olfactometer experiments. BBOB males were strongly attracted to a mixture of nonanal, decanal and geranylacetone, particularly to decanal alone, pointing out that this aldehyde is probably the most behaviorally active compound of the mixture. When pitting nonanal against decanal in the two Y-tube arms, males preferentially moved to the arm containing decanal, indicating that the one carbon difference in the chain length is sufficient to allow males to discriminate between the two aldehydes. Females, in turn, in odor vs blank experiments showed the strongest attraction to the 3-component blend and geranylacetone. Therefore, geranylacetone evoked a significantly different response in males than in females suggesting a different perception of volatiles in both sexes. The "anomalous" behavior of females, attracted by these synthetic stimuli but not by live insect (male or female) volatiles may be due to the much higher dose of the synthetic compounds tested relative to the amount of these compounds emitted by live individuals. This is in line with the lower number of olfactory sensilla found in females in comparison to males, as described above. Taken together, although aggregation tests in the field need to be performed, our results and the fact that BBOB individuals feed in groups suggest that these three compounds could also promote aggregation behavior in BBOB, with decanal being particularly important in the odor system of males and geranylacetone in females.

The headspace volatiles of the host plant *Q. suber* are mainly composed of saturated and monounsaturated 6-carbon aldehydes, alcohols, and esters, the green leaf volatiles GLVs that are released by most green plants (Hatanaka et al., 1987; Takabayashi et al., 1996) and whose production increases dramatically after mechanical or herbivore damage (Paré and Tumlinson, 1996). Other volatile chemicals emitted in minor amounts by freshly cut *Q. suber* branches include mono-, homo-, and sesquiterpenes. These results agree with the findings of Staudt et al. (2004), but are in contrast to others (Kesselmeier and Staudt, 1999; Loreto, 2002) who considered the holm oak *Q. ilex*, not *Q. suber*, the only emitter of monoterpenes. This inconsistency could be the result of a geographic variability in the overall capacity of *Quercus spp.* to emit volatiles, which may be related to past genetic isolation of populations, adaptations to local growth conditions, and/or hybridization between emitting and non-emitting oak species (Manos et al., 1999; Belahbib et al., 2001).

Most of the major GLVs identified from the host, (*E*)-2-hexenal, (*E*)-2-hexenol, 1-hexanol, (*Z*)-3-hexenyl acetate, and *n*-hexyl acetate evoked EAD responses in antennae of both sexes. In addition, in dual-choice bioassays, virgin females but not males were strongly attracted to the arm containing the natural blend of GLVs and the individual synthetic compounds (*E*)-2-hexenol, 1-hexanol, and (*Z*)-3-hexenyl acetate. In a previous work, these two alcohols in combination with (*Z*)-3-hexenol were found to attract individuals of the European cockchafer *Melolontha melolontha*, whereas the corresponding aldehydes and acetates were behaviorally inactive (Reinecke et al., 2002). Likewise, several studies have shown that GLVs emitted by foliage of green ash (*Fraxinus pennsylvanica* M.), white ash (*F. americana* L.) and Manchurian ash (*F. mandshurica*) and sesquiterpenes derived from the bark of stressed green ash trees attract EAB adults (Rodriguez-Saona et al., 2006; Crook et al., 2008; Crook and Mastro, 2010; Grant et al., 2010; 2011). Our results suggest that GLVs from the host, particularly (*E*)-2-hexenol, 1-hexanol, and (*Z*)-3-hexenyl acetate, may play an important role as attractants in the foraging/oviposition behavior of BBOB adults, particularly females.

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Captions to Figures

Fig. 1 Representative GC-EAD response on BBOB male antennae responding to (a) female volatiles collected during 24 h, and (b) 3-component blend of the synthetic compounds (100 ng each) 4: nonanal; 6: decanal; NA: nerylacetone; PHTH: phthalate; 10: geranylacetone. Numbers on compounds correspond to the entries in Table 2. IS = internal standard.

Fig. 2 Representative GC-EAD profile of BBOB female (a) and male (b) antennae responding to *Q. suber* volatiles collected during 24 h. Numbers on compounds correspond to the entries in Table 2. 2: (*E*)-2-hexenal; 3: (*Z*)-3-hexenol; 4: (*E*)-2-hexenol; 5: 1-hexanol; 6: (*Z*)-3-hexenyl acetate; 7: *n*-hexyl acetate; 23: (3*E*)-4,7-dimethyl-1,3,7-nonatriene.

Fig. 3 Behavioral responses of virgin BBOB males and females in a Y-tube olfactometer to volatiles of 3 live individuals and abdominal extracts (N=3) of both sexes. Number of insects attracted to odors vs blank (solvent control and air) was analyzed by $\chi 2$ goodness-of-fit test with Yates correction for continuity (*$P \leq 0.05$; ***$P \leq 0.001$). Bars with different letters indicate significant differences between ratios (test/control) of the treatments (one-way ANOVA followed by Tukey's HSD test; $P \leq 0.05$). N = number of responding individuals. Air vs air = control experiment.

Fig. 4 Behavioral responses of virgin BBOB males and females in a Y-tube olfactometer to nonanal, decanal and geranylacetone tested individually and in a 3-component blend (1:1:1 ratio) at the dose of 100 ng each. Number of insects attracted to odors vs blank (solvent) were analyzed by χ^2 goodness-of-fit test with Yates correction for continuity (*$P \leq 0.05$; **$P \leq 0.01$). Bars with different letters indicate significant differences between ratios (test/control) of the treatments (one-way ANOVA followed by Tukey's HSD test; $P \leq 0.05$). N = number of responding individuals.

Fig. 5. Competitive experiments in a Y-tube olfactometer of virgin male *C. florentinus* testing two synthetic odors and a 3 component blend (geranylacetone, nonanal, decanal) (100 ng each) against each other. Preferences for decanal (odor treatment 1) versus the mixture (blend 3 compounds) and nonanal (odor treatment 2) were analyzed by $\chi 2$ goodness-of-fit test with Yates correction for continuity (*$P < 0.05$). Bars with different letters indicate significant differences between ratios (odor treatment I/odor treatment II) of the two tests (one-way ANOVA followed by Student's t-test; $P \leq 0.05$). N = number of responding individuals. The same amount of hexane (10 µl) was applied to the odor treatments.

Fig. 6 Behavioral responses of virgin BBOB males and females in a Y-tube olfactometer to a blend of five synthetic GLVs ((*E*)-2-hexenal, (*E*)-2-hexenol, 1-hexanol, (*Z*)-3-hexenyl acetate and *n*-hexyl acetate, 1 µg each) and odors derived from volatile collections and leaf extract of the host plant *Q. suber*. Number of insects

attracted to odors were analyzed by $\chi 2$ goodness-of-fit test with Yates correction for continuity (*$P \leq 0.05$). Bars with different letters indicate significant differences between ratios (test/control) of the treatments (one-way ANOVA followed by Tukey's HSD test; $P \leq 0.05$). N = number of responding individuals.

Fig. 7 Behavioral responses of BBOB virgin females in a Y-tube olfactometer to the synthetic GLVs (*E*)-2-hexenal, (*E*)-2-hexenol, 1-hexanol, (*Z*)-3-hexenyl acetate and *n*-hexyl acetate (1 µg each) found in the host plant *Q. suber*. Number of insects attracted to odors were analyzed by $\chi 2$ goodness-of-fit test with Yates correction for continuity (*$P \leq 0.05$; **$P \leq 0.01$). Bars with different letters indicate significant differences between ratios (test/control) of the treatments (one-way ANOVA followed by Tukey's HSD test; $P \leq 0.05$). N = number of responding individuals.

Figure
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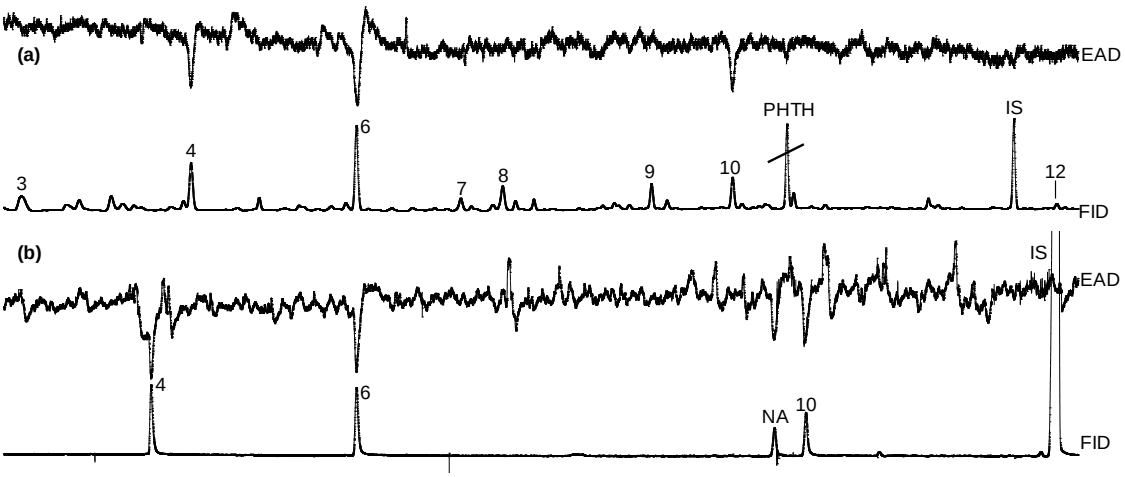

Fig. 1

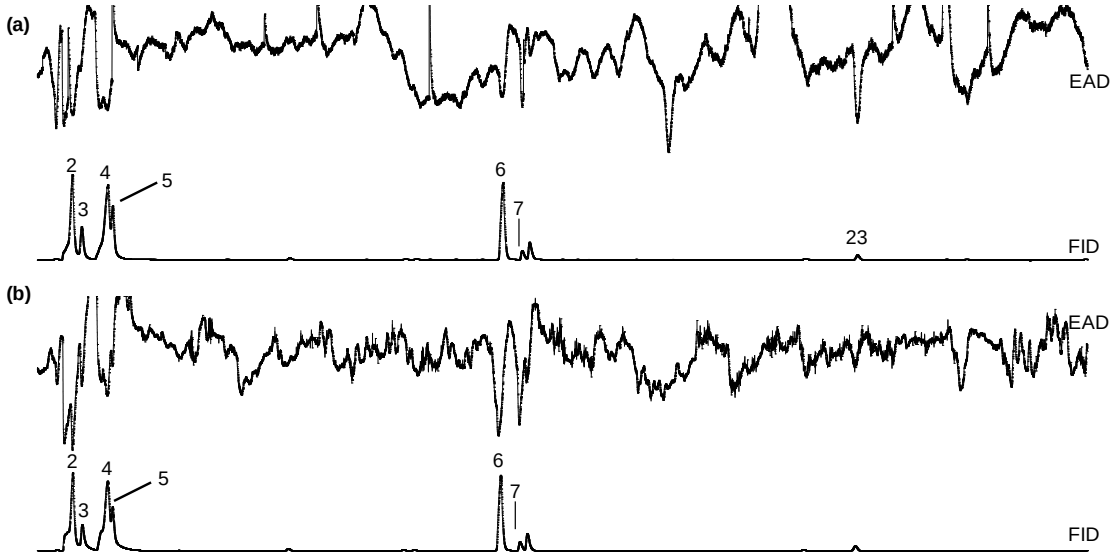

Fig. 2

Figure
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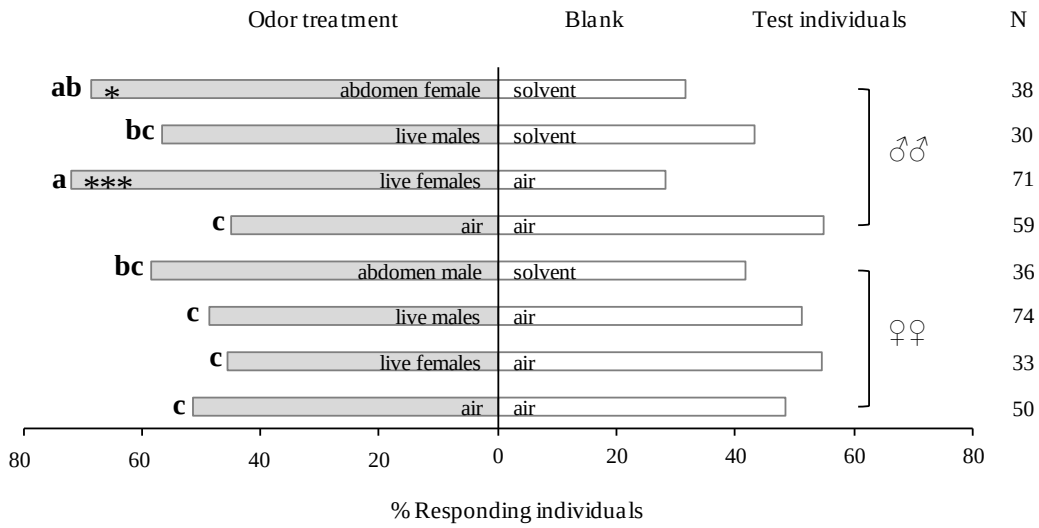

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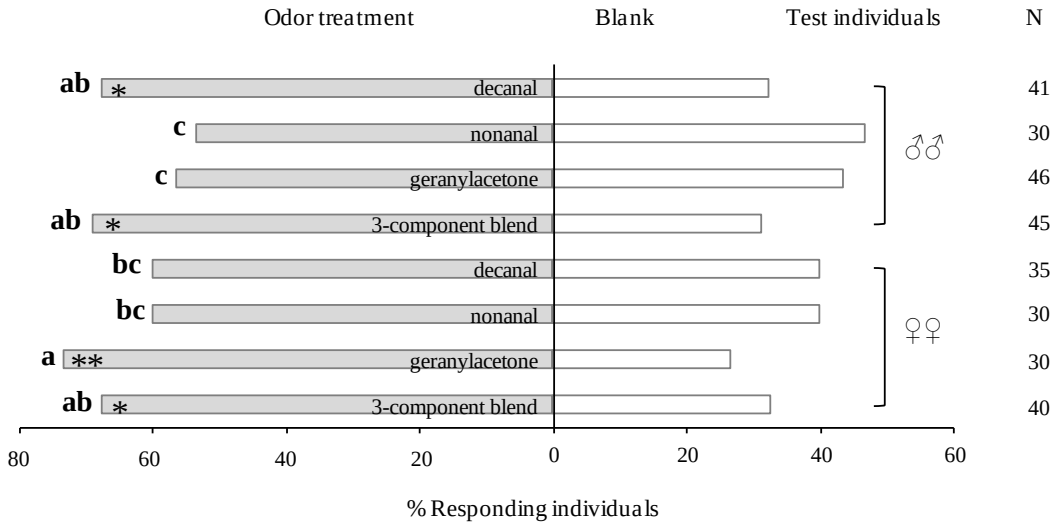
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Fig.4

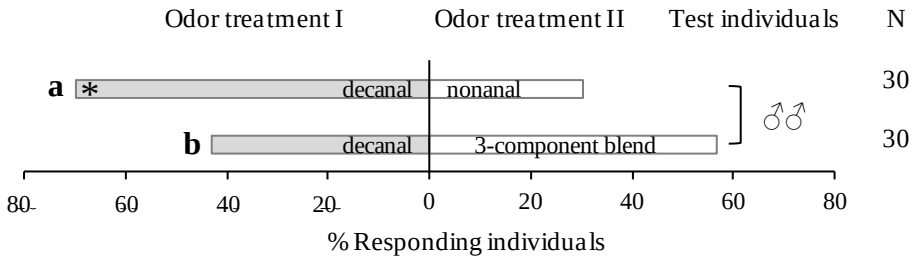
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Fig.5

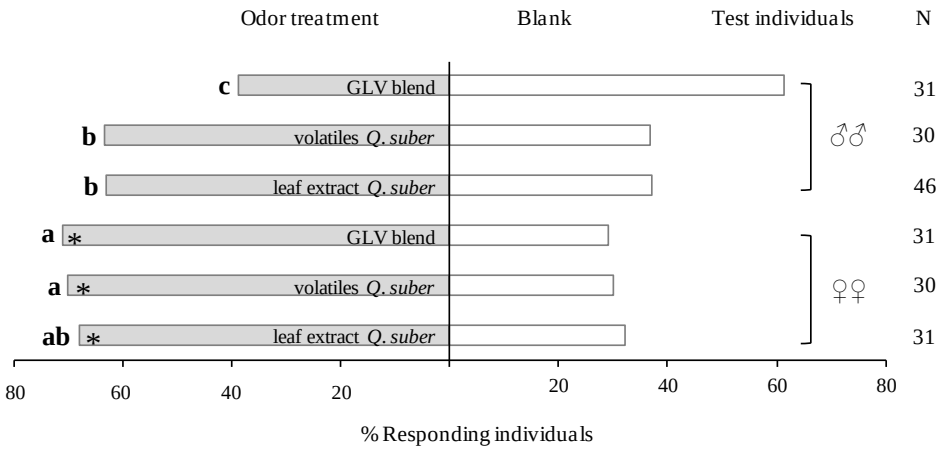
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Fig. 6

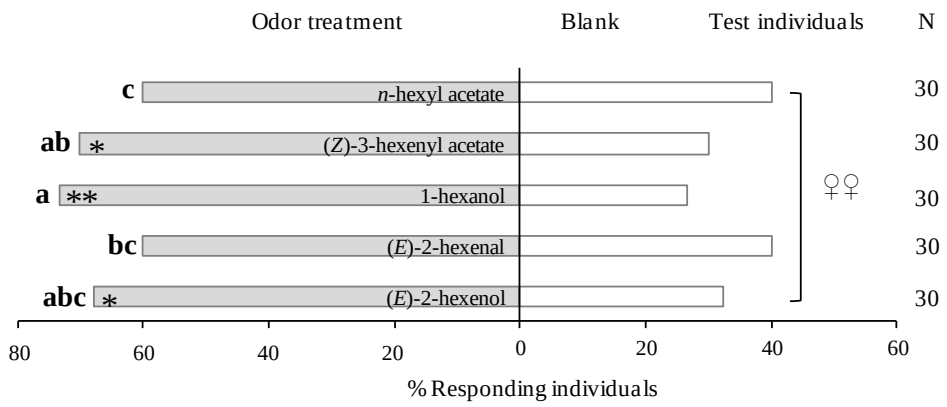
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Fig. 7

Table
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Table 1 Compounds detected in abdominal extracts[A] and volatiles[V] of BBOB males and females, identified by comparison of their mass spectra (MS) and retention indices (RI) with those of standards and/or reference databases

Entry	Compound	MS/RI[a]	M[b]	RI[c]
1	benzaldehyde[V]	ST/ST	106	963
2	6-methyl-5-heptene-2-one[V]	RD/RD	126	988
3	octanal[V]	ST/ST	128	1005
4	nonanal[A, V]	ST/ST	142	1103
5	2-phenylethanol[A]	ST/ST	122	1112
6	decanal[V]	ST/ST	156	1207
7	nonanoic acid[A]	ST/ST	158	1282
8	isobornyl acetate[V]	ST/ST	196	1289
9	tetradecane[V] ($C_{14}H_{30}$)	ST/ST	198	1399
10	geranylacetone[V]	ST/ST	194	1454
11	hexadecane[V] ($C_{16}H_{34}$)	ST/ST	226	1599
12	isopropyl dodecanoate[V]	ST/ST	242	1635
13	tetradecanoic acid[A]	ST/ST	228	1767
14	hexadecanoic acid[A]	ST/ST	256	1985
15	isopropyl hexadecanoate[V]	ST/ST	298	2025
16	henicosane[V] ($C_{21}H_{44}$)	ST/ST	296	2100
17	docosane[V] ($C_{22}H_{46}$)	ST/ST	310	2200
18	tricosane[A, V] ($C_{23}H_{48}$)	ST/ST	324	2300
19	11-methyltricosane[A, V] ($C_{24}H_{50}$)	RD/RD	338	2330
20	tetracosane[A, V] ($C_{24}H_{50}$)	ST/ST	338	2400
21	pentacosane[A, V] ($C_{25}H_{52}$)	ST/ST	352	2500
22	13-methylpentacosane[A, V] ($C_{26}H_{54}$)	RD/RD	366	2529
23	13-methylhexacosane[A, V] ($C_{27}H_{56}$)	RD/RD	380	2625
24	heptacosane[A, V] ($C_{27}H_{56}$)	ST/ST	380	2700
25	11,14-dimethylhexacosane[A, V] ($C_{28}H_{58}$)	RD/RD	394	2728
26	nonacosane[A, V] ($C_{29}H_{60}$)	ST/ST	408	2900
27	13-methylnonacosane[A, V] ($C_{30}H_{62}$)	RD/RD	422	2943
28	triacontane[A, V] ($C_{30}H_{62}$)	ST/ST	422	3000

[a] Type of identification by comparison of the MS and RI with those of standards (ST) or reference databases (RD).
[b] Molecular mass.
[c] On a HP-5MS (30 m × 0.25 mm ID × 0.25 μm) fused silica capillary column.

Table 2 Compounds detected in volatiles of *Q. suber* branches and leaf extracts, identified by comparison of their mass spectra and retention indices (RI) with those of standards and/or reference databases, and relative amounts to the most abundant compound (*E*)-2-hexenol

Entry	Compound	Rel. amount±SE[a]	M[b]	BP[b]	RI[c]
	Green leaf volatiles (GLVs) (C6)				
1	hexanal	10.0±4.1	100	41	813
2	(*E*)-2-hexenal	98.3±14.6	98	41	856
3	(*Z*)-3-hexenol	33.0±2.4	100	67	859
4	(*E*)-2-hexenol	100	100	57	870
5	1-hexanol	59.2±16.3	102	56	872
6	(*Z*)-3-hexenyl acetate	71.9±3.6	142	43	1009
7	*n*-hexyl acetate	11.5±1.5	144	43	1014
8	(*E*)-2-hexenyl acetate	18.6±3.4	142	43	1016
9	*(Z)*-3-hexenyl isobutyrate	0.7±0.2	170	67	1143
10	(*E*)-2-hexenyl isobutyrate	1.3±0.2	170	71	1150
11	(*Z*)-3-hexenyl 2-methylbutyrate	5.7±2.0	184	67	1232
12	(*E*)-2-hexenyl 2-methylbutyrate	1.6±0.5	184	57	1238
	Monoterpenes (C10)				
13	*α*-thujene	0.5±0.1	136	93	925
14	*α*-pinene	2.0±0.5	136	93	932
15	camphene	0.1±0.1	136	93	946
16	sabinene	1.2±0.3	136	93	972
17	*β*-pinene	1.3±0.3	136	93	975
18	limonene	0.3±0.1	136	68	1028
19	cineol	0.5±0.1	154	81	1030
20	(*E*)-β-ocimene	0.9±0.3	136	93	1047
21	γ-terpinene	0.5±0.1	136	93	1057
22	linalool	1.9±0.3	154	71	1100
	Homoterpenes (C11)				
23	(3*E*)-4,7-dimethyl-1,3,7-nonatriene	8.9±2.0	150	69	1117
	Sesquiterpenes (C15)				
24	*α*-cubebene	0.3±0.1	204	105	1352
25	copaene	0.4±0.1	204	161	1377
	Others				
26	nonane	0.1±0.1	128	43	900
27	(*E*,*E*)-2,4-hexadienal	0.8±0.1	96	81	909
28	*n*-pentyl acetate	0.4±0.1	130	43	912
29	benzaldehyde	0.9±0.1	106	77	959
30	vinyl hexanoate	0.8.0.1	142	60	983
31	γ-hexanolactone	0.9±0.1	114	85	1053
32	nonanal	0.8±0.2	142	41	1104

[a] Based on the relative areas by GC-MS.
[b] M: Molecular mass; BP: Base peak (m/z) of EI mass spectra.
[c] On a HP-5MS (30 m × 0.25 mm ID × 0.25 μm) fused silica capillary column.